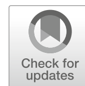
Chapter 5
Microbial Biosensors for Real-Time Monitoring of the Bioremediation Processes



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5.1 Bioremediation: An Eco-friendly Tool for Environmental Rehabilitation

Environmental pollution is one of the most crucial and commonly discussed issues for decades worldwide, yet the establishment of appropriate solutions or remedial measures for this problem is still in its infancy. Despite the seminal advancement in science and technology, the world is currently experiencing diverse adverse impacts of environmental pollution. Deliberate and accidental discharge of contaminants on large scales has amplified the health risks and environmental degradation equally affecting both developing and developed nations. According to the World Bank reports, the largest environmental cause of disease and premature deaths is pollution, with more than nine million premature deaths worldwide (Pollution 2021). This is 15 times higher than the deaths caused by wars and other forms of violence (Pollution 2021). Diverse physical, chemical and thermal approaches to mitigate the pollution or to restore the contaminated sites have been brought into action over the years. However, inherent limitations and drawbacks in these methods (e.g., high costs and production of toxic intermediates) have led scientists to shift towards novel environmental remediation methods such as biodegradation or bioremediation. Bioremediation involves biological systems such as microorganisms, their products or plants for the rehabilitation of contaminated soil or water. This eco-friendly method employs the naturally occurring enzymatic processes of the microorganisms and plants (phytoremediation) or sometimes a combination of both (rhizoremediation) to transform toxic pollutants into an innocuous state (Arora 2018). Depending on the site of application, bioremediation is of two types; ex situ and in situ. Bioremediation techniques have been applied in various ecosystems

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such as cleaning up groundwater, lagoons, sludge, oil spills, water streams, agricultural sites, and reclamation of sites contaminated with heavy metals, radioactive elements, petroleum and hydrocarbons (Goel et al. 2008; Bhatnagar and Kumari 2013; Arora 2018; Bhatt et al. 2021).

The ultimate goal of managing polluted sites is to transform them into a non-hazardous, pollutant-free site that is environmentally acceptable to be utilized for future purposes. Accordingly, monitoring the bioremediation process has become an indispensable aspect in environment research to evaluate the overall performance and to predict its outcome. Even though bioremediation is highly appreciated as an environmentally friendly and cost-effective tool in cleaning up contaminated platforms, it is imperative to clearly demonstrate its efficiency, reliability, reproducibility and predictability. Therefore, an interdisciplinary, systemic conceptual framework of monitoring the bioremediation process at each level is required for the successful implementation of the bioremediation setup. Once an appropriate bioremediation technique is chosen, regular monitoring has to be done to provide sufficient information for the optimization of the bioremediation process and to evaluate the efficacy for further scaling up of the treatment procedure. Therefore, the development of stringent and accurate monitoring protocols must be tailored to provide comprehensive details of the efficacy of the treatment process. The monitoring techniques for bioremediation must be constructed in a way that could address the following critical questions.

1. Economical—Is the chosen bioremediation technique is economically competitive with other methods?
2. Chemical—Were the targeted endpoints of cleaning the polluted site is achieved?
3. Sustainability—Is the bioremediation method employed environmentally and economically sustainable and could be reproduced in the future?
4. Engineering—Can the treatment process used be engineered and optimized further to use in a different context? Can the process be used to establish predictive models to extrapolate outcomes in another application?
5. Eco-toxicological—Did the treatment process transformed the polluted site completely into a harmless site? Is there any toxic effect or threat posed by bioremediation technique to humans or local biodiversity?
6. Biological involvement—What proportion of the biotic and abiotic factors contributed during the treatment process?

Considering the abovementioned facts, monitoring the bioremediation process can be performed concerning three different aspects, namely: (1) determining the efficiency of the pollutant degradation process, (2) assessing the survival and activity of degradative microbes, and (3) eco-toxicity assessment. The techniques that are used in environmental remediation are anticipated to provide efficient and accurate measurements to determine the overall efficiency of the bioremediation process.

5.2 Quantification of Pollutant Degradation by Non-microbial Tools

Remediation efficacy is usually quantified by the time-dependent endpoint measurement of the complete disappearance of the pollutant. This method is aided by the advancement of different analytical techniques to obtain mechanistic details of the degradation of specific pollutants, especially under in situ conditions (Pandey et al. 2009). Such analytical techniques can be advantageous or disadvantageous depending on the type of environment (the content of organic matter or clay in soil), nature of the contaminant or mixture of contaminants and other factors (Fig. 5.1). For example, Gas Chromatography (GC)/Flame Ionization Detection (FID) is preferable for identifying environmental contaminants as FID shows a linear response to a wide range of concentrations of the target. However, the sensitivity of this method reduces in the presence of oxidizable carbon compounds. Similarly, luminescence techniques are recognized as highly sensitive and selective tools for detecting microbial activity and the content of aromatic compounds. Nevertheless, the applicability of luminescence technologies for detection purposes is limited by the field conditions and the presence of multispecies oil-degrading microbial communities (Andreoni and Gianfreda 2007). One of the most effective

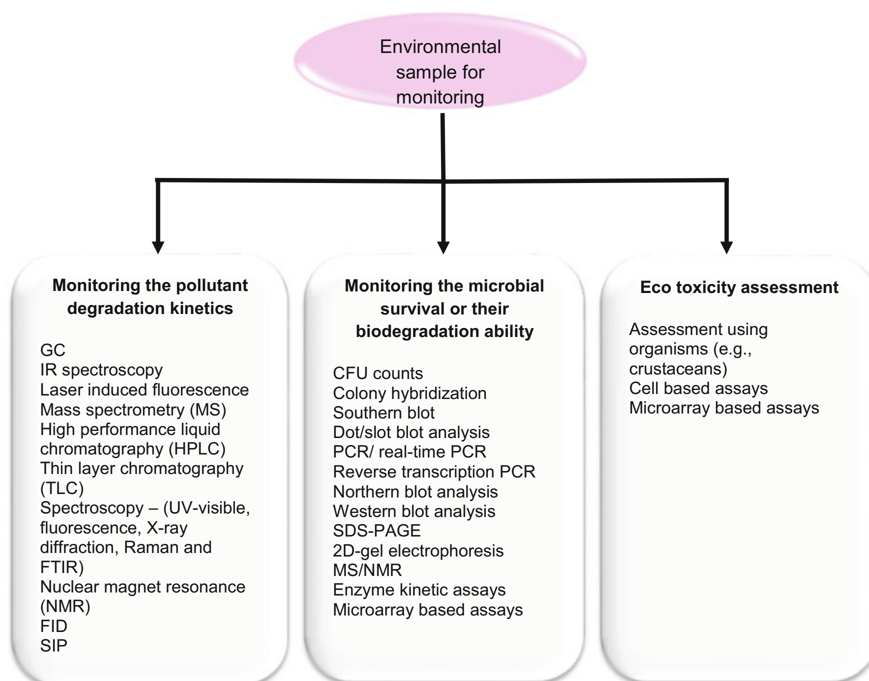

Fig. 5.1 Widely employed analytical techniques available to monitor the pollutant degradation process

techniques of monitoring the bioremediation process is the spectroscopic methods, among which UV-visible and Fourier-transform infrared spectroscopy (FTIR) methods are widely used. *Par excellence* of the spectroscopic analytical methods is due to their ability to rapidly monitor the degradation process while accurately and efficiently identifying the degradation intermediates (Pandey et al. 2009). Fluorescence spectroscopy is another analytical technique that is very sensitive in detecting aromatic pollutants, albeit the applicability of this method in complex mixtures is often minimal due to poor resolving features of the spectra (Gómez et al. 2004). To overcome the limitation of analyzing and quantifying complex mixtures, scientists have used a combinatorial approach involving infra-red (IR), fluorescence, synchronous luminescence spectrometry and GC techniques (Gómez et al. 2004).

Although the early analytical methods employed to monitor environmental remediation were largely depending on the kinetics of pollutant removal, the application of an eco-friendly analytical tool that utilizes survival and biodegradation abilities of microorganisms to quantify pollutant removal has shown a growing interest over the years. In this context, both the survival of the microorganism and the biodegradation ability as a function of time have been used to determine the effectiveness of the bioremediation process (Pandey et al. 2009). Cellular and molecular techniques are employed to assess microbial cell survival and their activity (Fig. 5.1). These techniques can be categorized into two broad sectors, namely: culture-dependent and culture-independent (Suyal et al. 2019a, b; Kumar et al. 2021). The culture-dependent method depends solely on obtaining the colony-forming unit (CFU) counts and is frequently utilized as a quick way to monitor the survival of a target microbe (Pandey et al. 2009).

Therefore, non-culturing technologies became more popular and equipped with modern molecular biology tools. To determine the survival of the target organism at the site of treatment, DNA isolated from samples is subjected to subsequent analysis (e.g., southern blot hybridization, dot/slot blot hybridization and PCR amplification, the latter being used for quantification of DNA). Positive amplification of the DNA sequence of interest indicates the survival of the target microorganism (Pandey et al. 2009; Debbarma et al. 2017). Similarly, the activity of the target organism can be monitored using transcriptome or proteome analysis. The latter employs enzyme assays and biochemical techniques such as Western blot, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), two dimensional (2D)-gel electrophoresis and liquid chromatography coupled to tandem mass spectrometry (MS/MS) via electrospray ionization source (Stenuit et al. 2008; Suyal et al. 2019a, b). However, limitations in extracting pure proteins from environmental samples and the tendency of proteins to structurally disorganize during the extraction process (protein denaturation) significantly affect the outcome of the study. Similarly, the activity of the organism can be studied at the RNA level using Northern blot, reverse transcription PCR (RT-PCR) or microarray analysis (Pandey et al. 2009; Kumar et al. 2019). High throughput microarray analyses can be used to monitor the catabolic potential of the targeted organism in real-time and have been used to monitor the bioremediation process in wastewater and other complex environments (Dennis et al. 2003). Although microarray-based assays provide the

advantage of high throughput, comprehensive and quantitative characterization of microbial communities, further extensive research has to be conducted to validate the applicability of this technique in diverse environmental samples (Zhou and Thompson 2002).

5.3 Limitations Associated with Conventional Monitoring Techniques

Research conducted over the years has clearly demonstrated the positive impacts of using non-microbial analytical methods to decipher and monitor the degradation of pollutants. However, quantitation of pollutants using such methods greatly depend on the extraction method of the pollutant as different extraction methods performed under various conditions may give inconsistent results. Therefore, different extraction methods were employed primarily based on the physio-chemical characteristics of the pollutant to enhance the extraction efficiency. These methods are mainly categorized as exhaustive and non-exhaustive extraction methods (Pandey et al. 2009). Another major drawback in using analytical methods for pollutant degradation is the inability to distinguish between biological vs. non-biological degradation. The final quantitation of the pollutant may vary due to the non-biological phenomena such as photolysis, wash off, leaching, diffusion and adsorption to the substrate, and thus may not completely reflect the full potential of the bioremediation process (Strand et al. 2003). As a solution, stable isotope probing (SIP) technology that relies on the content of stable isotopes in the molecule of interest can be applied. In this technique, fluxes of specific chemicals are traced in microorganisms by introducing a heavier stable isotope such as C^{13} to the microbial community (Panigrahi et al. 2018). Incorporation of the isotope into microbial cellular components such as nucleic acids can be detected after separation by gradient centrifugation and can be used as biomarkers in SIP technology. Widely used informative biomarkers include DNA, RNA or phospholipid fatty acids (PLFA). Nonetheless, SIP technology is based on the assimilatory process of the microorganisms and therefore, non-assimilatory processes such as co-oxidation fall outside the applicable framework of SIP technology. Furthermore, the use of SIP technology is restricted due to the necessity of the substrate to be labeled close to 100% for a successful density-based separation. Such labeling is expensive and requires long incubation times, and may not be suitable for regular monitoring purposes. Also, the synthesis of isotope-labeled DNA is limited by the replication efficiency of the organism. In contrast, RNA-SIP is a better responsive marker as the rate of RNA synthesis occurs at a high rate in active cells and will be efficiently labeled. However, in this context, efficient RNA extraction methods must be employed for the broad application of this method (Andreoni and Gianfreda 2007). Even though culture-dependent techniques such as colony hybridization has high selectivity and efficacy for assessing microbial activity and survival, these techniques have their inherent limitations in culturing

microbes, such as non-amenability for existing culturing protocols or the presence of viable but not culturable microbes (Pandey et al. 2009).

5.4 Eco-toxicity Assessment

The ultimate goal of the bioremediation process is to completely remove the hazardous waste or transform it into a harmless state such that the site could be reused in the future. Assessment of eco-toxicity in the pollutant site may not directly indicate the progress of the treatment method. However, eco-toxicity assessment paints the final picture of the bioremediation process by assessing whether the bioremediation process has positively impacted to reduce the toxicity imposed in the eco-system or not. At the end of an ideal bioremediation process, a significant reduction in the eco-toxicity of the target environment is anticipated. The most common eco-toxicity assessment assays employ luminescent marine bacteria, fungal biomass, shrimps, earthworms and crustaceans (Barajas-Aceves et al. 2002; Pandey et al. 2009). However, these assays require a longer time for evaluation as the toxicity is tested over a range of concentrations at different time points and may not be suitable for high throughput eco-toxicity assessment during the bioremediation process. Therefore, human cell line-based in vitro assays and microarray-based assays were developed. Furthermore, microarray-based analysis can be considered as a rapid, cost-effective and high throughput technique to study the catabolic responsiveness of an organism in the presence of a toxic chemical (Pandey et al. 2009).

Due to the limitations associated with conventional monitoring techniques, scientists have expanded the research to elucidate novel methods to assess environmental remediation. As a result, biosensors have emerged as a strong and versatile tool that could be engineered to get precise data in real-time.

5.5 Biosensors as a Powerful and Innovative Analytical Tool

A biosensor is defined as an analytical device that uses a biological molecule or living organism to sense the target molecule (e.g., sugars, proteins, hormones, pollutants, toxins, chemical compounds, antigens, enzymes, nucleic acids and microorganisms) (Hansen and Sørensen 2001; Bahadir and Sezgintürk 2015). The idea of using biological molecules for sensing the presence of the target molecule dates back to the 1960s where Clarks and Lyons used electrodes immobilized with glucose peroxidase to measure the levels of glucose in biological samples (Vigneshvar et al. 2016). Since then, numerous research efforts lead to the discovery of highly accurate and selective biosensors that could be utilized in various

applications, including medical diagnosis, environmental monitoring, drug discovery, and food quality monitoring. In contrast to the conventional methods, improved detection limits, selectivity, accuracy, and sensitivity of biosensors have led to continuous development in this realm and resulted in remarkable advances to bring about highly sensitive biosensors for high-throughput real-time monitoring purposes. Biosensors have two basic components, namely: the biological component and the transducer (Mehrotra 2016). The biological material or biomimetic functions as the recognition molecule, which is either in intimate contact with the transducer or integrated within the physiochemical transducer or transducing microsystem (Korotkaya 2014). The transducer or the detector element transforms the physico-chemical changes into processable signals (optical, piezoelectric, electrochemical, electro-chemiluminescence, etc.) that are proportional to the amount of target molecule–bioreceptor interactions (Bhalla et al. 2016). These signals are produced due to a change in proton concentration, emission of light, emission of heat, production and uptake of gases such as oxygen and ammonia and many other mechanisms which result from sensing an analyte. This process of energy transformation is called signalization. The selection of biological material to develop a biosensor depends mainly on the analyte to be detected. In addition, biochemical specificity, storage, operational and environmental stability also plays a vital role in this selection (Lim et al. 2015). Biosensors can be classified into two broad categories based on the sensing biological material and the type of transducer used (Fig. 5.2). An ideal biosensor must possess properties such as specificity, sensitivity, reliability, portability and the ability to function in optically opaque solutions, real-time analysis and simplicity in operation (Bhalla et al. 2016). Biosensors are typically comprised of an electronic system including a signal amplifier, a processor and a display in addition to the bio-recognition site and the bio-transducer components (Bhalla et al. 2016). The processor functions as a reader which analyses signal modifications, amplify and present the detecting signal. The fluctuation in the signal entirely depends on the analyte-bioreceptor interactions and truly reflects inherent bio-sensitivity to the analyte (Bhalla et al. 2016). High selectivity and specificity of biological materials combined with the processing power of microelectronic devices give rise to versatile biosensors that could be employed in various aspects for accurate detection and monitoring purposes. However, it is necessary to ensure that the analyte reaches the site of reaction in the biological material. The biological component and the electrical signal can be manipulated further by amplification and processing to improve the quality of biosensors to meet the market requirements. The analytical capabilities of a biosensor can be further increased by miniaturization and improved processing systems (Bhalla et al. 2016).

Different types of biosensors have been introduced over time to monitor the bioremediation process to assess its effectiveness (Fig. 5.2). Biosensors have emerged as an attractive monitoring tool and have gained much attention in contrast to conventional techniques. Portability, high selectivity, rapid and on-site point of care monitoring favored the use of biosensors over other conventional methods for monitoring purposes. Among the bio-recognition elements indicated in Fig. 5.2, enzymes have been widely used for monitoring purposes. Nevertheless, the tedious

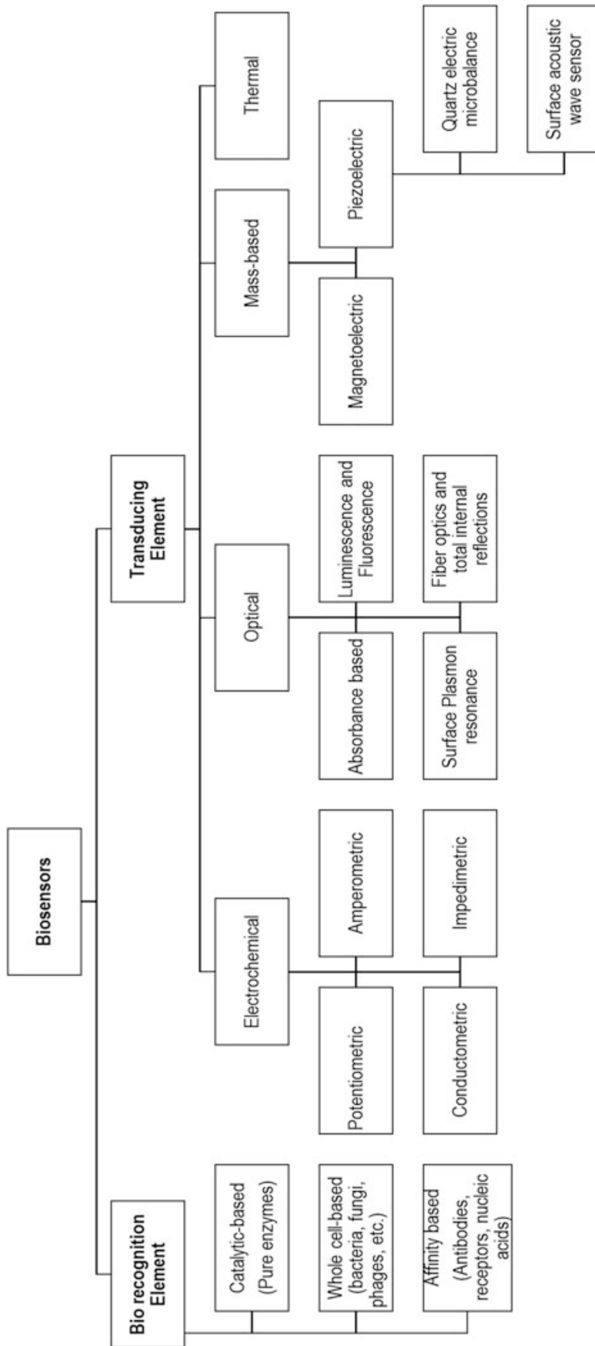

Fig. 5.2 Classification of biosensors

purification process, the necessity for co-enzymes or multi enzymes to give a detectable signal and the associated high costs in producing enzyme-based biosensors have led scientists to search for alternatives. Moreover, when considering enzymes and antibodies, maintaining their stability, specificity, activity and/or affinity in different environmental conditions is quite difficult (Park et al. 2013). Hence, scientists have presented microbial biosensors as convenient and cost-effective biosensors that could be utilized under a range of environmental conditions for monitoring the bioremediation process. Microbes can be cultivated on a large scale by following a simple cell culturing process. Besides, all the necessary co-factors for an enzymatic reaction reside inside the cell and comparably more stable and demonstrates more capacity to tolerability in harsh environmental conditions. Furthermore, microbes can be handled and manipulated easily in a way that can enhance the expression of a necessary pathway to augment the emitted signal or even can be manipulated to endure harsh environmental conditions.

This chapter is dedicated to provide a comprehensive overview of employing microbial biosensors for monitoring the bioremediation process and describe detailed mechanistic information about how synthetic biology, molecular biology, chemistry and engineering have interfaced to design a resourceful and ideal microbial biosensor.

5.5.1 Design and Fabrication of a Microbial Biosensor

Microbes have gained more research attention in developing biosensors as they have the potential to target a wide range of elements, and even they can be easily manipulated to enhance the specificity towards the substrates. Evidence from numerous research efforts suggests that genetic engineering of microbes is comparably much easier and seems to be better controlled and tailored to give the best-desired outcome than using plants or mammalian cells and other types of biosensors (Lim et al. 2015). There are two mechanisms by which microbial biosensors induce a specific reaction during the biosensing process; inhibition of cellular respiration or alteration of cell metabolism in the presence of an analyte of interest (Xu and Ying 2011). As a consequence of the change in microbial cellular metabolism induced by the targeted analyte, gene expression of the sensing elements is also changed. This change in gene expression is being detected and/or quantified using a microbial biosensor (Fig. 5.3). The sensing elements of a biosensor mainly consist of regulator genes and bio-recognition genes/reporter genes. The regulatory genes control the differential expression of the bio-recognition element based on the presence or absence of the target analyte. The bio-recognition gene or the reporter gene functions by converting the biological response into a detectable or measurable signal. Given the fact that the expression of the biorecognition gene is maneuvered by the regulator gene, the requirement of a promoter could be eliminated. Therefore, the design of a genetically engineered microbial biosensors may consist of only a regulator gene and a bio-recognition gene without a promoter. The resulting recombinant genes can

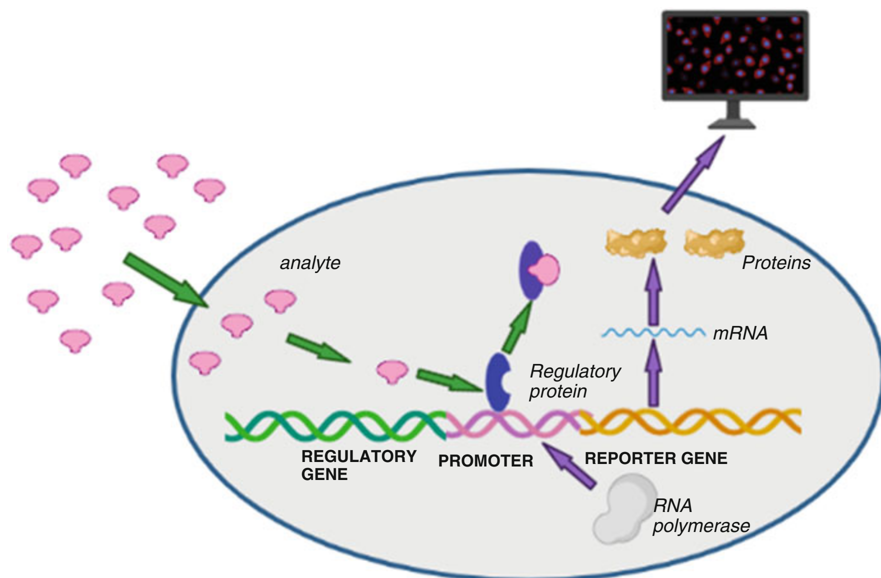

Fig. 5.3 Schematic illustrating the mechanism of a typical microbial biosensor. The diagram shows a negative regulatory mechanism where binding of the analyte to the regulatory protein frees the promoter region for RNA polymerase to express the reporter gene. Finally, the expressed reporter protein is detected by a transducer

then be cloned into the microbial host either by direct integration into the chromosome or by cloning into an appropriate plasmid vector and transforming it to the host. In the presence of the target compound or the analyte, the regulator stimulates the promoter, which in turn performs the transcription and translation of the reporter gene to a protein that can be detected as an electrochemical, chemiluminescent, colorimetric or fluorescent signal. The generated signal can be either a qualitative or a quantitative signal depending on the design of the biosensor. Based on the aforementioned facts, it is clear that the excitability of regulatory genes towards the analyte of interest is the key determinant of the specificity and sensitivity of a microbial biosensor (Bilal and Iqbal 2019). Therefore, proper selection of the regulatory genes, host strains and a suitable detection technique govern the successful utilization of a biosensor.

5.5.2 Host Strain

The selection of an appropriate bacterial strain is the most crucial step in designing a biosensor. The selected microbial strain must have the substrate specificity for the targeted analyte to detect the presence of it by eliciting a cellular response. Furthermore, there are several ways in which the microbial traits can be successfully

translated into a routine assay format to fulfill different analytical and monitoring requirements, which include but are not limited to monitoring the bioremediation process and assessing the toxins and other chemicals in the environment. Microorganisms that have been widely utilized as biosensors generally do not have complex multicellular structures and are unicellular organisms. Immobilized cells can act as both sensing components and generators of the recognition signals. Bacteria and yeast are being widely used as the immobilized cell type in biosensors (Xu and Ying 2011).

Identification of the best-suited host strain for the biosensor improves its specificity, sensitivity and time-response (Gui et al. 2017). Selection of the host strain primarily depends on the target analyte, bio-sensing elements, sensitivity, specificity and the detection mechanism. A typical example of a highly sensitive biosensor is the biosensor hosted on *Pseudomonas putida* DOT-T1E, which was found to be the best strain for monitoring sites that are heavily contaminated with toxic organic compounds. *P. putida* DOT-T1E possesses this unique property as it is evolutionary optimized to survive in environments that are highly concentrated with such toxins whilst showing high substrate specificity to several contaminants such as antibiotics, toluene and flavonoids (Espinosa-Urgel et al. 2015; Gui et al. 2017). Another biosensor for the detection of Ni^{2+} in drinking water has been designed using the wild type *Escherichia coli* strain designated as *E. coli* TD2158. This strain has shown the highest level of sensitivity and activity for Ni^{2+} compared to other strains of *E. coli*, such as *E. coli* W3110-based biosensors that utilized the same mechanism of detection. Consequently, *E. coli* TD2158 was identified as the best host strain giving the highest sensitivity, to design the biosensor for detecting Ni^{2+} in drinking water (Gui et al. 2017). The selection of the host strain also depends on the type of analyte to be detected. For example, microbial biosensors have been constructed using *Acinetobacter baylyi* ADP1 for the detection of a broad range of alkanes and alkenes in water and soil. The use of *E. coli* as the host for this purpose has been restricted due to the poor accessibility and emulsifying capability of *E. coli* for oils. In contrast, *A. baylyi* ADP1is naturally adherent to the oil-water interface while emulsifying the minerals and oils and thus, it is an excellent strain for constructing a whole-cell biosensor for detecting a range of alkanes and alkenes in water, seawater or in oils (Gui et al. 2017).

Genetic approaches and gene modifications play a significant role in bringing successful microbial biosensors to execution. Genetic engineering provides solutions to most of the frequent drawbacks associated with the use of wild-type strains and helps to retain an improved selectivity and sensitivity within the biosensor. More commonly, bacteria are genetically engineered by incorporation of a specific reporter gene to respond to the chemicals present in the sample or physiological stress through synthesis of a reporter protein such as green fluorescence protein (GFP), luciferase, β-galactosidase, etc. (Xu and Ying 2011).

5.5.3 Reporter Genes

The performance of a microbial biosensor for the detection of environmental contaminants strongly depends on the reporter gene/s chosen to detect the genetic response for the contaminants and the type of regulatory protein associated with the promoter. A reporter gene is capable of converting its biological response into a measurable signal (e.g., electrical, optical, electrochemical, etc.) which determines the sensitivity and selectivity of the biosensor (Gui et al. 2017). Widely used reporter genes that can be successfully incorporated into microbial biosensors include *cat* (encodes bacterial chloramphenicol acetyltransferase), *cfp* (encodes coral fluorescence proteins), *lux* (encodes bacterial luciferase), *luc* (encodes firefly luciferase), *gfp* (encodes green fluorescent protein) and *lacZ* (encodes β-galactosidase) (Bullock and Gorman 2000; Hansen and Sørensen 2001; Chong and Ching 2016; Gui et al. 2017).

E. coli lacZ that encodes the protein β-galactosidase is one of the best-studied and most frequently used reporter genes in biosensors. *LacZ* exhibits unique advantages for analyte detection, such as employing convenient and sensitive colorimetric or fluorescent methods that utilize readily available chemiluminescent and electrochemical substrates. In addition, low detection limit (as low as 2 fg), ultra-high sensitivity, and an extensive dynamic detection range are some of the key advantages of using the *lacZ* reporter gene (Gui et al. 2017). The reporter gene bacterial luciferase (*lux*) catalyzes the oxidation of long-chain fatty aldehydes and reduces flavin mononucleotides to form the corresponding fatty acid and fructosamine in the presence of oxygen and produces bioluminescence as the reaction output (Xu and Ying 2011). *Lux* genes isolated from various bacterial strains (e.g., *luxCDABE* operon from *Vibrio fischeri*, *luxCDABFE* operon from *Photobacterium leiognathi,* and luciferase coding *luxAB* from *Vibrio harveyi*) have been widely used as reporter genes in biosensor constructs (Hansen and Sørensen 2001). Many researchers have used fiber optic technology to detect light emission from *lux* biosensors (Hansen and Sørensen 2001). Visualization of the light emission from the luciferase can be observed without disruption of the bacterial cell. However, *lux* is rarely used in mammalian cell-based biosensors due to thermal instability and protein dimerization which can lead to false interpretations (Gui et al. 2017). *Gfp* that encodes GFP has been widely used as a marker in many bacterial biosensor constructions. GFP is a very stable fluorescent protein that can be excited with UV or blue light and the fluorescence can be detected without bacterial cell lysis. Further, it does not require the addition of exogenous substrates or ATP to generate the signal (Xu and Ying 2011). Therefore, unlike *lacZ*, GFP is not limited by the accessibility of the substrate (Hansen and Sørensen 2001). Some variants of GFP have half-lives of more than 24 h, while the others have a half-life of ~40 min (Hansen and Sørensen 2001). This could be advantageous because GFP variants with an extended half-life can be produced even from weak promoters or in cells with low metabolic activity (Hansen and Sørensen 2001). Another advantage is the use of GFP variants with shorter half-life in transient (real-time/time-dependent) gene expression studies for the detection

of various analytes (He et al. 2019). Therefore, to detect dynamics and to facilitate rapid degradation inside the microbial cells, these fluorescent reporters have been destabilized to shorten their half-lives. However, applications of this approach are limited by the very low signal intensities generated by the GFP variants with short half-life. A possible solution to overcome this challenge would be the development of a trans-timer using a destabilized GFP with another GFP variant called RFP (red fluorescent protein), which can detect the dynamics of gene expression in cells (He et al. 2019). When the target gene is 'on', the destabilized GFP expresses rapidly before the RFP expression turns on and when the target gene turns 'off', the GFP that was expressed will rapidly degrade leaving only the red signal by RFP. Therefore, in a biosensor, dynamic monitoring can be done using the ratio of green to red colour emitted by the GFP based trans-timer (He et al. 2019). Use of GFP has been limited due to the cost of equipment, such as flow cytometers and fluorimeters which are necessary to analyze the fluorescence signal. Another disadvantage is that the detection limit of GFP is higher than that of both β-galactosidase and luciferase (Hansen and Sørensen 2001).

Other reporter genes used include eukaryotic *luc* from the firefly *Photinus pyralis* (Hansen and Sørensen 2001). The firefly *luc* reporter gene has frequently been incorporated into mammalian and bacterial cells due to its high sensitivity and linearity over a broader range of analyte concentrations (Gui et al. 2017). At the same time, the use of *luc*, especially with mammalian cells, could overcome the thermally labile nature and dimeric protein interferences generally associated with bacterial *lux* (Gui et al. 2017). Another example is c*rtA*, a gene involved in carotenoid biosynthesis. This reporter gene allows the detection of target analyte calorimetrically through the naked eye upon introducing it into a biosensor. When applied to a sample, *crtA*-based biosensors can change the color of the culture media from yellow to red without the addition of a supporting substrate and therefore, is considered a good choice for rapid detection of the target analytes in emergencies (Gui et al. 2017). A potential disadvantage associated with *crtA* is that the production of carotenoids is often affected by the metabolic fluxes of the host microorganism. This can interfere with the color intensity and the time required for color development when using the biosensor (Chong and Ching 2016). Therefore, a promising solution to produce an intense color development with little influence from the metabolic fluxes would be the use of *cfp* as the reporter gene (Chong and Ching 2016). It is considered a favorable candidate as the coral fluorescence protein not only enables a visible colorimetric change but also shows a minimal dependency on the amount of metabolites available (Chong and Ching 2016). Table 5.1 is a summarization of the reporter genes widely employed in microbial biosensors.

5.5.4 Regulatory Proteins

The gene regulatory proteins are one of the major components on which the performance of a biosensor depends (Gui et al. 2017). They are the proteins that

Table 5.1 Reporter genes that are frequently used in microbial biosensors (Chong and Ching 2016; Gui et al. 2017)

Gene	Detection method	Advantages	Disadvantages
lux	Luminescence	Easy measurement, rapid response	Thermal labiality, requirement for the substrate O_2
luc	Luminescence	High sensitivity, rapid response, thermal stability	Requirement for the substrates; O_2 and ATP, low permeability
gfp	Fluorescence	No substrate requirement, high stability	High cost of equipment, low sensitivity, lag-time for stable fluorescence, auto-fluorescence
lacZ	Luminescence, fluorescence, colourimetry, electrochemistry	High stability, wide variety of detection methods, detection by naked eyes	Substrate dependence (e.g., X-gal), low permeability
crtA	Colorimetry	Detection by naked eyes, no substrate requirement	Activity is affected by the metabolic fluxes of the host organism

influence the regions of a DNA molecule that are transcribed by RNA polymerase during transcription. These proteins, which include transcription factors, help control the synthesis of proteins in cells. They possess complex interactions with the target analytes or the contaminants of interest. These interactions are critical for the specificity and sensitivity of a biosensor (Gui et al. 2017). These regulatory proteins have been reported to respond to a wide array of compounds (e.g. sugars, vitamins, secondary metabolites, metal ions, amino acids and other lipid metabolites) and serves as a large reserve of biological components that can be utilized for designing in vivo biosensors (Shi et al. 2018).

Biosensors that have utilized the function of these regulatory proteins have shown higher selectivity, higher detection ranges and enhanced sensitivity when compared to conventional biosensors. The binding of the analyte to the regulatory protein induces a significant conformational change, thus activating/inhibiting the expression of the reporter gene (Raut et al. 2012). Therefore, the function of the regulatory protein can be either positive or negative in terms of the activation of the promoter for the expression of the reporter gene (Raut et al. 2012). In negative regulation, the regulatory protein is bound to the operator/promoter region, inhibiting the expression of the reporter gene. When the analyte is bound to the regulatory protein, it dissociates from the operator/promoter region, subsequently allowing RNA polymerase to carry out the downstream reporter gene expression (Fig. 5.2). In positive regulation, the analyte-regulatory protein complex binds to the operator/ promoter region facilitating binding of the RNA polymerase to carry out the expression of the reporter gene (Raut et al. 2012). For example, an *E. coli* biosensor for the detection of insecticide CPF (chlorpyrifos) has been designed by using a CPF inducible locus *chpAB* found in *Sinorhizobium meliloti*. CPF biosensors utilize a gene designated *chpR*, a cadC family transcription regulator, as a positive regulator for the *chpAB* operon, which is involved in the detection of CPF (Whangsuk et al. 2010). A brief comparison of a few microbial biosensors designed so far is given in Table 5.2.

Table 5.2 Widely used whole-cell biosensors for the detection of environmental pollutants (Gui et al. 2017)

Host strain	Reporter gene	Target analyte	Detection sensitivity
E. coli	*luxCDABE*	Arsenic	0.74–69 μg/l
E. coli	*lacZ*	Arsentate	<10 μg/l
D. radiodurans	*lacZ* *crtI*	Cadmium	1–10 mM 50 nM–1 nM
E. coli	*gap*	Chromate	100 nM
E. coli	*gfp*	Zinc Copper	16 μM 26 μM
E. coli	*luc*	Benzene, Toluene and Xylene	40 μM
E. coli	*luxAB*	Benzene, Toluene and Xylene	0.24 μM
P. putida	*luxAB*	Phenol	3 μM
B. sartisol	*luxAB*	Naphthalene and Phenanthrene	0.17 μM
E. coli	*luxAB*	C_6–C_{10} Alkanes	10 nM
E. coli	*luxCDABE*	Tetracycline	45 nM
S. typhimurium	*lacZ*	Single-stranded DNA	10 nM

5.5.5 *Microbial Immobilization Techniques*

A microbial biosensor is designed in such a way that host microorganisms are in close contact with the transducer. Therefore, the use of the proper immobilization technique is necessary to establish the required connectivity between the cell and the transducer which need to be placed in close proximity (Lei et al. 2006). An immobilization technique should preserve cell viability and functionality, and the immobilization matrix must provide mechanical stability to prevent cell leakage. Furthermore, such methods must ensure the efficient access of the analyte molecules into the cells (Lobsiger and Stark 2019). Chemical immobilization techniques such as covalent binding, cross-linking, and physical immobilization techniques such as adsorption, entrapment have been widely used in the fabrication of a microbial biosensor (Lobsiger and Stark 2019; Ganesan and Vasudevan 2021).

The covalent immobilization technique relies on the formation of strong covalent bonds between the different functional groups present on the microbial cell wall and the transducer. These functional groups include amine, carboxylic, sulfhydryl and tosyl groups. Harmful chemicals and harsh reaction conditions used in covalent binding have a negative impact on cell viability; thus covalent binding is rarely employed with viable microbial cells (Lei et al. 2006). Cross-linking is a process which involves the formation of a network of cells by interconnecting the functional groups in the outer membrane by chemicals such as glutaraldehyde and cyanuric chloride. Similar to covalent binding, cross-linking too may affect the cell viability. (Lei et al. 2006).

Physical immobilization techniques are in wide use when dealing with the viable cells, as it has the minimal interference with the native structure and function of the microorganism. Adsorption is considered to be the simplest form of physical

immobilization technique. Microorganisms are immobilized due to different adsorptive interactions such as ionic, polar and hydrogen bonds (Lei et al. 2006). Entrapment is another widely used physical immobilization technique. During entrapment, cells are retained using dialysis, using a filter membrane or polymer (Lei et al. 2006). The polymers used in entrapment include hydrogels such as agarose, LB agar and alginate (Lobsiger and Stark 2019). However, low sensitivity limits the use of entrapment as the microbial immobilization technique (Lei et al. 2006). Lyophilization or freeze-drying is another physical technique used in the immobilization of microorganisms during the fabrication of a biosensor. This is a low-temperature dehydration process where water from a frozen sample is sublimed in a vacuum, thus preserving the integrity of the microbial cell (Lobsiger and Stark 2019).

5.6 Diversity of Microbial Biosensors

Tremendous research efforts equipped with the advancement in microbial biotechnology, micro-engineering and synthetic biology have led to the development of promising and more futuristic microbial biosensors with enhanced performance. This large pool of microbial biosensors can be categorized based on different criteria. In this chapter, the microbial biosensors are broadly categorized into three major categories in terms of their signal transducers utilized, namely: electrochemical, optical and microbial fuel-cell type biosensors.

5.6.1 Electrochemical Microbial Biosensors

Electrochemical microbial biosensors are the most widely available type of microbial biosensors and have been reported to have the highest sensitivity among all the available microbial biosensors. They mainly consist of a working electrode, microorganisms as a transducer layer for detection and a signal recording equipment (Lim et al. 2015). These types of biosensors exploit the respiratory electrochemical pathways of microorganisms. The analyte interacts with a component in the microorganism's respiratory pathway, which acts as an electron shuttle or a mediator. This interaction leads to an inhibition of the transmission of signals causing a change in the electrochemical potential, which is subsequently detected by the transducing mechanism (Ikeda and Kano 2001; Yang et al. 2018). To improve the sensitivity, externally supplied redox-active mediators which can get reduced in the cell can be used to amplify the signal via transferring electrons through the system (Gupta et al. 2019). As mentioned, electrochemical microbial biosensors are capable of providing specific quantitative or semi-quantitative analytical information with the use of biological recognition elements and can be further classified based on the mechanism used by the transducer to detect the signal (Xu and Ying 2011).

5.6.1.1 Types of Electrochemical Biosensors

(a) Voltametric Microbial Biosensors

This is the most versatile form of electrochemical biosensor type for the detection of chemical compounds. Each electric signal generated through a current and a voltage difference is recorded and correlates with a corresponding sample. Voltametric approaches can provide high selectivity and measurability via the position and density of the peak current signal. Low detection speeds and the requirement of complex components for the process are the potential limitations associated with these types of biosensors (Lim et al. 2015).

(b) Conductometric Microbial Biosensors

Conductometric microbial biosensors detect chemicals by the variation in conductivity of a sample solution caused by target analytes. Detection happens via the consumption or production of ions by the transducers. The conductance measurements are highly sensitive and can detect the target chemicals rapidly (Lim et al. 2015). In particular, they can easily be miniaturized as they do not require a reference electrode. Even with high sensitivity, the detection of solution conductance is considered to be nonspecific because the variation in conductivity can be affected by the electrical charge (Xu and Ying 2011). Microorganism-based conductometric biosensors are primarily being used in the detection of microbial toxicity in the dairy industry (Xu and Ying 2011).

(c) Amperometric Microbial Biosensors

Amperometric microbial biosensors monitor the concentration of the chemical by recording the current signal through the sample at a fixed potential with respect to a reference electrode (Lim et al. 2015). The corresponding current is obtained by the oxidation or reduction of electroactive species at the surface of the electrode. In particular, amperometric microbial biosensors have been recorded to provide greater sensitivity. Most of the biosensors designed to measure biological oxygen demand (BOD) belong to this category (Xu and Ying 2011).

(d) Potentiometric Microbial Biosensors

A Potentiometric microbial biosensor consists of either an ion-selective electrode or a gas-sensing electrode (Xu and Ying 2011). This approach uses the potential difference from a reference electrode and thus requires three electrodes as two working electrodes and a reference electrode. The need for a reference electrode for stable and accurate sensing is a limitation associated with potentiometric microbial biosensors. This type of biosensors shows a higher selectivity and sensitivity for the target chemical (Lim et al. 2015).

5.6.1.2 Application of Electrochemical Biosensors in Environmental Monitoring

Electrochemical microbial biosensors have proven their capability for the identification and analysis of different target compounds due to their simplicity, portability and cost-effectivity. Several attempts have been made to exploit the electrochemical sensors' potentialities to detect emerging contaminants in the environment, which include pesticides, antibiotics, heavy metals and perfluorinated compounds. Since electrochemical biosensors typically utilize the intrinsic electron transfer ability of microorganisms, the signal can be enhanced by simply supplementing electron mediators externally without the need for any genetic alterations (Gupta et al. 2019). A wide range of electrochemical microbial biosensors has been constructed to detect and monitor many environmental pollutants and parameters. These include BOD, toxins such as 3,5-dichlorophenol (DCP) and trichloroethylene, herbicides, pharmaceuticals, heavy metal ions such as Cu^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, As^{3+} and Zn^{2+}, and anions like sulfide (Table 5.3). Microorganisms used in these biosensors include *Shewanella oneidensis*, *Saccharomyces cerevisiae*, *Bacillus subtilis*, *E. coli*, *Chromobacterium violaceum*, *Thiobacillus thioparus* and *Pseudomonas* sp. (Pham et al. 2015; Yang et al. 2018; Gupta et al. 2019). A recently discovered group of bacteria called "exoelectrogens" are widely employed to develop electrochemical biosensors. Exoelectrogens have the ability to transfer electrons outside of the cell. Thus, their intracellular electrochemical pathways can be linked to an extracellular transducing mechanism (Yang et al. 2018).

Among the listed microorganisms, *E. coli* is identified as the most widely utilized organism in biosensors. A typical example includes the development of a cadmium sensing biosensor for on-site monitoring of water, seawater and soil samples. The biosensor has been designed by fusing the cadmium responsive promoter of *E. coli* to a promoterless *lacZ* that encodes the enzyme β-galactosidase. An electrochemical assay based on the activity of β-galactosidase is used to quantify the cadmium level. Enzymatic conversion of the substrate p-aminophenyl-β-D-galactopyranoside (ONPG) to p-aminophenol (ONP) generates a current signal which can be detected electrochemically. Under anaerobic conditions, this electrochemical biosensor is reported to have a sensitivity to Cd^{2+} concentrations as low as 25 nM in water and 5 μM in soil (Shin 2011). *S. oneidensis* MR-1 is an example of an exoelectrogen that can connect its internal electrochemical pathways to an external circuit. Here, the electric current flow is subjected to change based on the type and the concentration of the environmental pollutant. Yang et al. (2018) have developed an electrochemical bacterial biosensor exploiting this ability of *S. oneidensis* MR-1 for the detection of DCP levels in the water. In the presence of DCP, the produced electric current decreases in a concentration dependent manner (Yang et al. 2018). The cyanobacterium *Anabaena variabilis* provides a suitable biological system to detect the presence of photosynthesis-inhibiting herbicides. In photosynthetic organisms, photosystem II (PSII) harvests light energy via an electron transfer chain which ultimately oxidizes water to produce O_2. Photosynthesis-inhibiting herbicides

Table 5.3 Widely used electrochemical microbial biosensors used in environmental monitoring

Analyte/parameter monitored	Microorganisms utilized	Detecting/transducing mechanism
BOD	*B. subtilis*	Electrochemical (amperometry)
	C. violaceum R1	Electrochemical (amperometry)
	Mixed culture including *Geobacter* sp.	Bioelectrochemical
3,5-dichlorophenol (DCP)	*S. oneidensis* MR-1	Bioelectrochemical
	S. cerevisiae S288C	Electrochemical (amperometry)
	E. coli ATCC 25922, *B. subtilis* CGMCC 1.1086, *S. cerevisiae* S288C	Electrochemical (amperometry)
4-chlorophenol, phenol	*S. cerevisiae* S288C	Electrochemical (amperometry)
Trichloroethylene	*Pseudomonas* sp. ASA86	Electrochemical (potentiometry)
Herbicide (Diuron)	*A. variabilis*	Electrochemical (amperometry)
	Chlamydomonas Reinhardtii	Electrochemical (amperometry and potentiometry)
Herbicide (Atrazine)	*A. variabilis*	Electrochemical (amperometry)
Pesticides (Ametryn and Acephate)	*E. coli* ATCC 25922, *B. subtilis* CGMCC 1.1086, *S. cerevisiae* S288C	Electrochemical (amperometry)
Pharmaceuticals (Omeprazole, lansoprazole, naphthoflavone and methylcholanthrene)	*Arxula adeninivorans* G1212/ YRC102	Electrochemical (amperometry)
Cu^{2+}	*S. cerevisiae* S288C	Electrochemical (amperometry)
	E. coli ATCC 25922	Electrochemical
	E. coli ATCC 25922, *B. subtilis* CGMCC 1.1086, *S. cerevisiae* S288C	Electrochemical (amperometry)
Cd^{2+}	*S. cerevisiae* S288C	Electrochemical (amperometry)
	E. coli ATCC 25922	Electrochemical
	E. coli ATCC 25922, *B. subtilis* CGMCC 1.1086, *S. cerevisiae* S288C	Electrochemical (amperometry)
Ni^{2+}	*S. cerevisiae* S288C	Electrochemical (amperometry)
Pb^{2+}	*S. cerevisiae* S288C	Electrochemical (amperometry)

(continued)

Table 5.3 (continued)

Analyte/parameter monitored	Microorganisms utilized	Detecting/transducing mechanism
	E. coli ATCC 25922	Electrochemical
Zn^{2+}	*E. coli* ATCC 25922	Electrochemical
As^{3+}	*S. oneidensis*	Bioelectrochemical
Sulfide	*E. coli* BL21	Voltammetry

competitively inhibit the electron transfer chain and thereby decrease the current generated (Fig. 5.4). Artificial redox mediators like quinone can be utilized to measure the electric current generated through this system (Tucci et al. 2020).

The concentration dependent inhibition of the current generation has been observed in the presence of two photosynthesis-inhibiting herbicides, diuron and atrazine. A three-electrode system, where the immobilized *A. variabilis* containing biosensor acts as the active electrode, has been utilized to obtain the electrochemical measurements. In the presence of atrazine, the electric current generated is decreased in a concentration dependent manner. In the presence of diuron, the electric current is completely inhibited due to diuron being a potent photosynthesis inhibitor (Tucci et al. 2020).

5.6.2 *Optical Microbial Biosensors*

These are the biosensor devices that make use of principles of optics for the transduction of a biochemical interaction into a detectable output signal (Xu and Ying 2011). Optical microbial biosensors are developed by coupling the ability of a microorganism to recognize a certain analyte (bio-recognition sensing element) with an optoelectronic transducer system (Gupta et al. 2019). The visual signal can be the result of bioluminescence, chemiluminescence, fluorescence or chromogenic detection (Axelrod et al. 2016; Bae et al. 2018). The two elements are frequency coupled via genetic modification by placing a reporter gene under the control of an analyte-specific promoter. Thus, the reporter gene is only expressed in the presence of the targeted analyte (Gupta et al. 2019). Optical microbial biosensors offer advantages such as flexibility and resistance to electrical noise. Optical fibers, as optical wave-guides, have been largely used in optical microbial biosensors due to their low cost, small size and flexible geometry. The optical fiber-based microbial biosensors can be easily taken to the field for on-site monitoring (Xu and Ying 2011).

5.6.2.1 Bioluminescent Microbial Biosensors

Bioluminescent microbial biosensors measure the change in luminescence emitted by microorganisms (Su et al. 2011). They are mainly employed for risk assessment

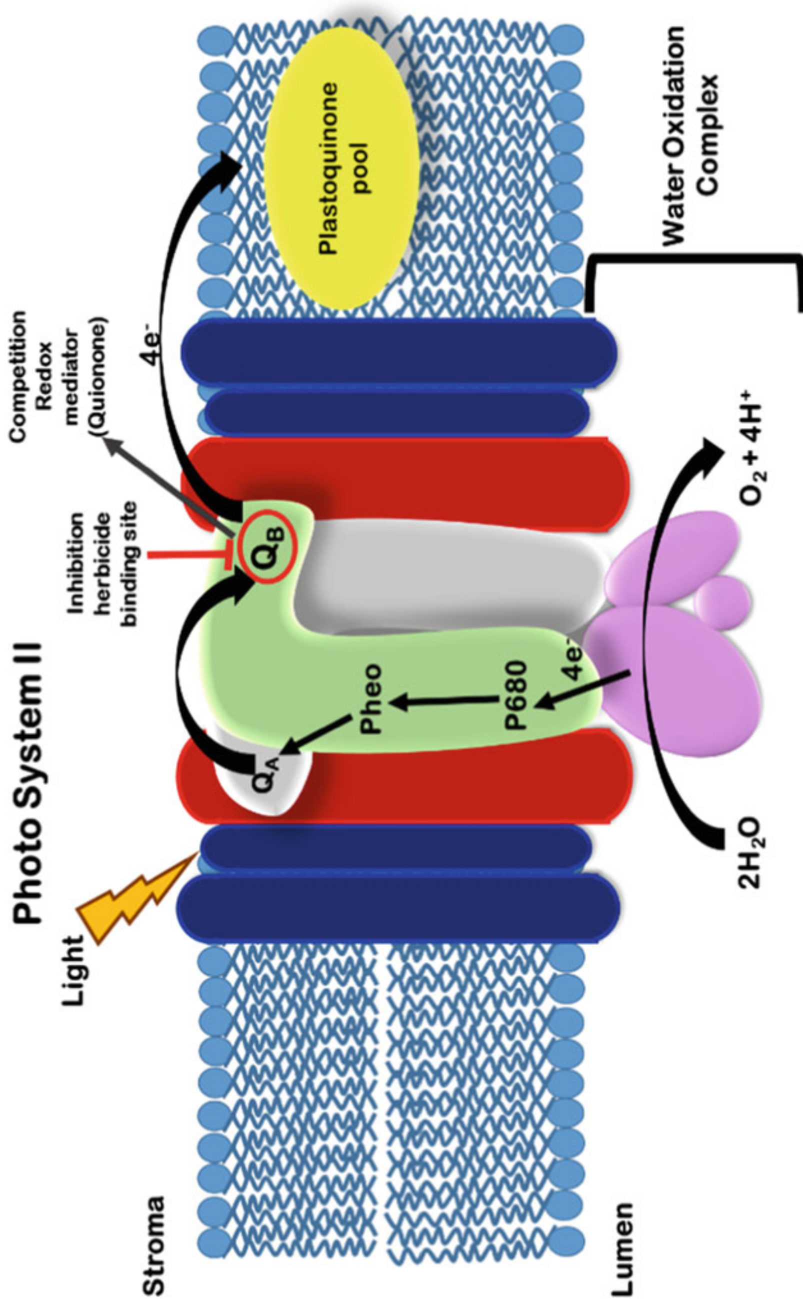

Fig. 5.4 Mechanism of action of photosynthesis-inhibiting herbicides in PSII. Electrons produced via the oxidation of water into O_2 are transported by the P680 protein. Naturally, the present electron acceptor plastoquinone accepts the electrons at the active site Q_B. An artificially provided redox mediator such as quinone can also competitively accept electrons through the same active site. Photosynthesis-inhibiting herbicides competitively bind the active site and inhibit the electron transfer chain

and environmental monitoring, especially for toxicity measurements and heavy metal detections. The emission of light by the microbial communities is mainly determined by the bioavailable fraction of the detected contaminant and therefore, this method is considered a reliable and efficient tool as a biosensor (Lim et al. 2015). Several forms of bioluminescent bacteria have been used in the development of bioluminescent biosensors, including natural bioluminescent bacteria and genetically modified light-emitting bacteria. These biosensors can be produced as single-chip, low-power, rugged, inexpensive components and can be deployed in a variety of non-laboratory settings. However, they may report having a lower efficiency due to the inherent problems associated with the light-emitting systems, which can be improved through genetic modification (Xu and Ying 2011). The bacterial luciferase encoded by *lux* and the eukaryotic luciferase *luc* from *Photinus pyralis* have been successfully served as reporter genes in a variety of bioluminescent microbial biosensors (Su et al. 2011).

5.6.2.2 Fluorescent Microbial Biosensors

Fluorescence occurs in some microbial cells when an external light source is applied. At low analyte concentration, the fluorescence emission intensity is directly proportional to the analyte concentration (Xu and Ying 2011). Based on the detection mode, fluorescent microbial biosensors can be divided into two categories, in vivo and in vitro (Su et al. 2011). In vivo biosensors make use of genetically engineered microorganisms with a transcriptional fusion between an inducible promoter and a reporter gene encoding a fluorescent protein. The Green fluorescent protein (GFP), which is encoded by *gfp*, is one of the most popular tools used in the in vivo fluorescent microbial biosensors due to its attractive stability and sensitivity. The fluorescence emitted by GFP can be conveniently detected with the use of modern optical equipment with little or no damage to the host system (Su et al. 2011).

5.6.2.3 Colourimetric Microbial Biosensors

Colorimetric microbial biosensors involve the generation of colored compounds that generates a signal which can be measured and correlated with the concentration of analytes (Su et al. 2011). These biosensors indicate the presence of the analyte by a visible color change of the microbial cell. Unlike in fluorescence or luminescence biosensors, colorimetric microbial biosensors generally do not need any special equipment for detection under light-shielded conditions and can be monitored by the naked eye both in the laboratory and the field (Fujimoto et al. 2006). The reporter genes which have been used widely in colorimetric biosensor include *lacZ*, *crtA* and *cfp* (Gui et al. 2017). *LacZ* from *E. coli* encodes the enzyme β-galactosidase, which splits its substrate X-gal into a blue colored product which can be detected by the naked eye. The colour intensity is proportional to the level of enzyme activity within a certain range thus, permits its use in a colorimetry based microbial biosensor to

detect the analyte of interest (Shin 2011). Similarly, enzymes such as alkaline phosphatase and horseradish peroxidase are also being used to generate the detection signals in different colorimetric microbial biosensors (Shin 2011). Recently, carotenoid-based colourimetric biosensors have been developed with the use of reporter genes *crtA* and *crtI*, which have the additional advantage of substrate independent color developing ability or the detection of the target analyte (Shin 2011).

5.6.2.4 Optical Microbial Biosensors in Environmental Monitoring

Optical microbial biosensors have been optimized to monitor the quality and toxic levels in the water. These biosensors can assess BOD, heavy metals like Hg, Pd, As, Cu and Zn, organic pollutants such as formaldehyde and methyl parathion, as well as herbicides and pesticides (Table 5.4). The microorganisms that are being used for the development of such biosensors include *S. cerevisiae*, *E. coli*, *Sphingomonas* sp., *Chlamydomonas reinhardtii* and *Dictyosphaerium chlorelloides* (Axelrod et al. 2016; Bae et al. 2018; Gupta et al. 2019). For example, a simple microbial biosensor has been developed using the bacterium *E. coli* that employs naked-eye detection of color change for the on-site detection of phenolic compounds in water and soil. The bio-recognition is mediated by a plasmid harboring the *β-galactosidase* gene fused with the phenolic responsive *CapR* promoter. This biosensor has shown a significant sensitivity to phenolic compounds and can respond in concentrations range from 0.1 μM to 10 mM (Shin 2011). Another colorimetric biosensor for arsenic detection

Table 5.4 Widely used optical microbial biosensors for environmental monitoring

Analyte/parameter monitored	Microorganisms utilized	Transducing mechanism
BOD	*S. cerevisiae*	Chemiluminescence
Organic solvents including formaldehyde	*E. coli* TV1061	Bioluminescence
	E. coli DPD2794, DPD2544 and TV1061	Bioluminescence
Ammonium Hydroxide	*E. coli* TV1061	Bioluminescence
Endocrine destructive agents including 17-β-estradiol (E2), 17α-ethynylestradiol (EE2), diethylstilbestrol (DES) and estrone (E1)	*E. coli* DPD2794, DPD2544 and TV1061	Bioluminescence
	S. cerevisiae	Bioluminescence
Pesticides (Diuron, Simazine, Atrazine)	*C. reinhardtii*	Fluorescence
Herbicide (Simazine)	*Dictyosphaerium chlorelloides* Dc1M	Fiber optic–luminescent O_2 transducer
Mercury	*E. coli* TV1061	Bioluminescence
Pb^{2+}	*E. coli* DH5α	Fluorescence
Cu^{2+}	*S. cerevisiae*	Colorimetric

has been developed with the use of the reporter gene *crtA*, which expresses spheroiden monooxygenase (Shin 2011). The host strain used is an engineered strain of *Rhodovulum sulfidophilum* with a deleted *crtA* locus which appears in yellowish due to the accumulation of yellow spheroiden. The biosensor has been constructed by cloning the arsenite resistance operon from *E. coli* into a plasmid containing *crtA* and transforming it into the host strain. This operon consists of an operator/promoter region and a repressor gene *arsR*. The presence of AsO_2^- induces dissociation of the repressor protein from the operator, thus allowing the expression of spheroiden monooxygenase. This enzyme catalyzes the formation of reddish spheroiden from yellowish spheroiden. Therefore, in the presence of AsO_2^-, a color change from yellow to red could be observed by the naked eye. The reported limit of the detection of AsO_2^- by this biosensor is 5 μg/L (Su et al. 2011).

In addition to the colorimetric biosensors, fluorescent and bioluminescent microbial biosensors have played a significant role in monitoring metal contaminations. The bacterial luciferase or *lux* is a widely used reporter gene in the development of luminescent microbial biosensors. Expression of the *lux* genes in microorganisms can be controlled in a constitutive or inducible way. For example, in India and Vietnam, a luminescent-based bacterial biosensor has been developed and deployed in the field for the assessment of groundwater samples contaminated with arsenic. This biosensor can detect the analyte with more than 90% accuracy and has been applied on a large scale for the environmental monitoring of arsenic. This *E. coli* DH5α based biosensor has been developed with the *luxCDABE* reporter gene of *Vibrio fischeri* cloned with the arsenic resistant operon (*ars*) of a wild-type *E. coli*. The operator/promoter region and the *arsR* (negative regulator gene) of the *ars* operon are cloned with the reporter gene *lux*, which expresses bacterial luciferase only in the presence of the target analyte arsenic. This process generates a luminescent signal which can be detected quantitatively, within an arsenic concentration range of 0.74–60 μg/L (Sharma et al. 2013). Similarly, *V. fischeri* based bioluminescent microbial biosensor has been shown promising results for rapid determination of common environmental pollutants (Su et al. 2011). Another biosensor for the detection of heavy metal concentrations in wastewater was designed with the host organism *Acinetobacter sp*. employing the reporter genes *luxCDABE* (Su et al. 2011). Additionally, a bioluminescent biosensor with *Pseudomonas fluorescens* has been designed to detect the fraction of naphthalene present in the soil (Su et al. 2011).

Similarly, in vitro fluorescent whole-cell biosensors are designed and successfully applied to monitor environmental pollutants such as heavy metals and O_2 to assess the BOD levels in water (Su et al. 2011). For example, an Ag^+ and Cu^{2+} sensitive biosensor has been constructed using a two-component (plasmid) system consisting of an Ag^+ sensor and a regulator from bacterial *sil* operon coupled to a detector (Sharma et al. 2013). The membrane-bound protein SilS from *E. coli* J53 detects the Ag^+ ions, which then activates the secondary protein SilR by transphosphorylation. Phosphorylated SilR protein thus becomes an activator that activates the promoters of the *silE* and *silABC*. These two promoters have been separately cloned upstream of a promoterless *gfp* thus, creating two plasmids named

pRADEK.1 and *pRADEK.2*, respectively. These two plasmids containing one of the two promoters with *gfp* transformed into the host *E. coli* J53 can be used as a fluorescence biosensor to qualitatively and quantitatively detect Ag^+. Due to the close homology of the *sil* operon with the copper resistance genes, this biosensor may be successfully applied to detect Cu^{2+} in a similar way to Ag^+ (Sharma et al. 2013).

A microbial biosensor has been developed to monitor the levels of Cu^{2+} in water, utilizing a modified *S. cerevisiae* BY4742 strain. The modification involves two genetic changes in the AMP pathway of purine synthesis, which results in the production of a colored pigment in the presence of Cu^{2+} ions. The intensity of the color correlates with the concentration of Cu^{2+} in the medium. Here, the *ADE2*, which codes for AIR (5′-phosphoribosylaminoimidazole) carboxylase, is knocked out. In the absence of the enzyme, AIR gets accumulated in cells and subsequently oxidized into a red-colored pigment in the presence of O_2. Hence, the cells appear red in color. The second modification is to place the *ADE5,7*, which encodes GAR (5′-phosphoribosylglycinamide) synthase, under the control of the CUP1 promoter, which is induced in the presence of Cu^{2+}. GAR synthase catalysis the first step of the AMP pathway. Thus, the pathway is only initiated when Cu^{2+} is present. Consequently, the red color pigment is only produced in the presence of $Cu^{2+,}$ while in the absence of Cu^{2+}, the pathway is not initiated, and the cells remain white (Vopálenská and Palková 2015).

5.6.3 Microbial Fuel-Cell Type Biosensors

Microbial Fuel Cells are novel and promising tools in environmental biotechnology. They can be considered as devices that can convert chemical energy into electrical energy through catalytic reactions present in the electroactive microbes. Therefore, this type of biosensors can generate electricity as the original signal by bio-degradation of organic matter, i.e., the catalytic activity of microorganisms converts chemical energy to electric energy in response to the target analyte. While there are many applications of MFCs, they have been widely used in the construction of whole cell-based environmental biosensors.

The basic structure of an MFC consists of anodic and cathodic compartments separated by an ion-exchange membrane (Fig. 5.5). The anode and the cathode are connected via an external circuit. In addition to the anode, the anodic chamber contains a culture of electroactive bacteria in a medium rich with substrate organic compounds. The bacteria catalyze the oxidation of organic substrates and produce electrons and positively charged ions such as H^+, K^+ and Na^+. The electrons are captured by the anode, and the external circuit conducts them to the cathode. The ion exchange membrane facilitates the transfer of cations from the anodic chamber to the cathodic chamber to balance the charges. In the cathodic chamber, oxygen accepts the electrons and protons to produce water (Jung et al. 2007; Cui et al. 2019). The catalytic rate of the conversion of chemical energy to electric energy can be affected

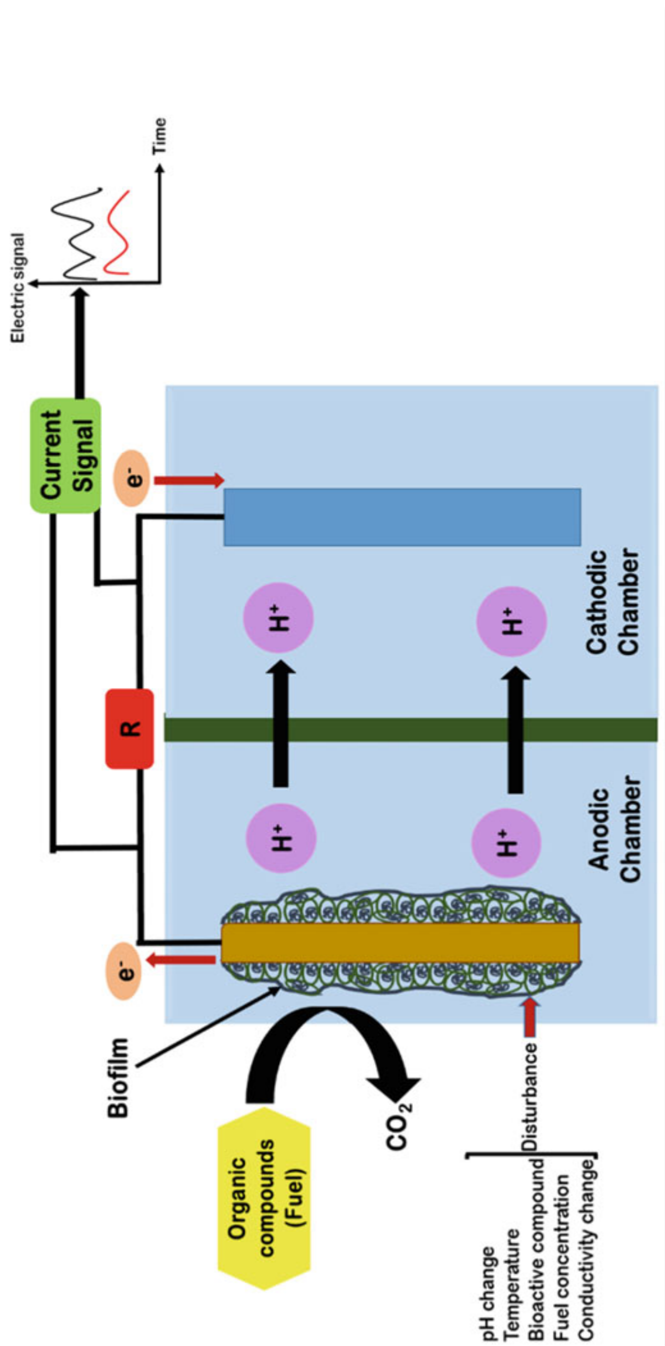

Fig. 5.5 Schematic of a microbial fuel cell. In the anodic chamber that contains microorganisms, the organic substrates are oxidized to produce electrons which are conducted to the cathodic chamber via an external circuit. Next, O_2 accepts the electrons to produce water. Oxidation of organic substrates in the anodic chamber also gives rise to cations which are transferred into the cathodic chamber across the ion exchange membrane

by various environmental parameters, which can lead to changes in the electron flow through the MFC, subsequently altering the electric current produced. Thus, the apparatus can be refined to construct a biosensor that measures such environmental parameters. In such a biosensor, the microorganisms in the anodic chamber would act as the receptor, which recognizes the changes in the environmental parameters. Instead of having a culture of bacteria, a biofilm containing bacteria can also be utilized. The anode which captures the electrons plays the role of the transducer. Typically the relationship between the electric current generated and the changes in the environmental parameters is considered to be linear (Cui et al. 2019).

5.6.3.1 Microbial Fuel-Cell Biosensors in Environmental Monitoring

Microbial biosensors have been developed using MFCs to detect a wide range of environmental parameters such as BOD, COD, heavy metal ions, and toxic compounds in water, as well as the activity of other microbes by utilizing biofilms, single bacterial cultures or mixed bacterial cultures (Table 5.5). The bacterial species that have been widely utilized in the development of MFC-based biosensors include, *Azospirillum*, *Acinetobacter*, *Ocillibacter*, *Shewanella loihica*, *Shewanella frigidimarina*, *Thermincola carboxydiphila*, *Pseudomonas aeruginosa*, *Ochrobactrum intermedium*, *Citrobacter freundii*, *Clostridium acetobutylicum* and *E. coli* (Cui et al. 2019; Gupta et al. 2019). For example, MFC-based biosensors have been used to detect toxic environmental agents. As mentioned, they utilize microbial metabolism as the driving force for the conversion of chemical energy into electrical energy. Therefore, the MFC output depends on the viability and activity of the bacterial cells (Sun et al. 2015). In contrast to the other microbial biosensor types, the MFC-based biosensor demonstrates long-term stability due to the self-healing property of the biofilm. In addition, the requirement of a transducer is eliminated in this biosensor as MFC is a self-powered device. However, MFC-type biosensors have certain limitations such as low substrate efficiency and low sensitivity due to the complicated biofilm (Fang et al. 2020).

Table 5.5 Frequently used microbial fuel-cell biosensors in environmental monitoring

Analyte/parameter monitored	Microorganisms utilized
BOD	*S. loihica* PV-4
	T. carboxydiphila, *P. aeruginosa*, *O. intermedium*, *S. frigidimarina*, *C. freundii*, *C. acetobutylicum*
Toxicity (avermectins (AVM), ivermectin (IVM), tetracyclines, heavy metals)	Mixed culture including *Azospirillum*, *Acinetobacter, Pseudomonas*, *Ocillibacter*
Cd^{2+}	*S. loihica* PV-4
Formaldehyde	*Shewanella oneidensis* MR-1

5.7 Advantages, Limitations and Future Challenges of Biosensors

Live cells offer the advantage of qualitative and quantitative analysis of a specific compound by emitting a signal as a response to their regular homeostasis process. Therefore, in contrast to conventional analytical techniques, microbial biosensor-based assays are undoubtedly much simpler and can be carried out without the need for expensive equipment. For example, hands-on demonstration carried out in public using the *E. coli* Arsr-LuxAB reporter assay provides evidence for the successful application of microbial bioreporter assays even by non-experts to get quality and accurate results in few hours (Van Der Meer and Belkin 2010). Moreover, unlike in enzyme-based biosensors, microbial biosensors do not exploit pure enzymes and hence, the stringent and costly purification steps can be excluded, and the entire reporter-sensor unit compacted in a self-replicating cell can be produced in a simple culturing step. In addition, enzymes and antibodies may subject to denaturation or inactivation during the extraction and purification process (Reshetilov et al. 2010). Such limitations can be overcome by the use of microbial biosensors. Modern advancements are more oriented to adapt a multi-well format for bioassays to achieve high throughput real-time monitoring. Therefore, microbes provide an ideal platform for miniaturizing the biosensors for high throughput monitoring whilst maintaining excellent accuracy in measurements required for better sample screening procedures. Furthermore, microbial biosensors come with the unique advantage of providing information pertinent to ecotoxicological safety endpoints of a particular contaminant in a site. This is because microbes elicit a specific response to the bioavailable fraction of the compound of interest in the sample (Van Der Meer and Belkin 2010). Given the strong analytical potential owned by microbes coupled with the cost-effectiveness, ease of handling, less technical hurdles, better stability in harsh environments and amenability for genetic manipulation to obtain pre-determined bioanalytical properties make microbial biosensors an ideal analytical tool to monitor environmental contaminants (Lim et al. 2015).

Although microbial biosensors provide many advantages in real-time monitoring of the bioremediation process, they do have a few drawbacks and limitations (discussed below) that need to be addressed in the near future.

5.7.1 Environmental Safety Concerns

Microbial biosensors utilize living microorganisms. Some of these microorganisms have been genetically modified to suit the needs of the monitoring process. Introducing a genetically modified microorganism may pose a risk to the environment as it can harm or disrupt the existing microbiome of the site, which may eventually disturb the ecological balance. Strict regulatory measures have been imposed restricting the use of genetically modified microbial biosensors in qualified

laboratories and contained environments. Thus, precautions should be taken when microbial biosensors are utilized so as not to introduce any destructive microorganisms into the environment. Furthermore, the possible malignant effects of the microbes used in biosensors must be thoroughly researched.

5.7.2 Non-target Interaction and Poor Signal Quality

Non-target interaction is commonly associated with microbial biosensors, which utilize electrochemical detection and transducing mechanisms. This is because the microbial cells can interact with many chemical species such as ions and organic compounds found within the environment other than the targeted analyte. This can lead to false-positive measurements as well as high background noise leading to poor signal quality and reduced specificity of the biosensor (Gupta et al. 2019). Using appropriate controls such as multi strain assay or gas phase assays in the bioreporter assay may minimize the complications associated with chemical mixtures (Van Der Meer and Belkin 2010). The signal quality of an optical microbial biosensor depends on the expression of the reporter genes. Even in the same microorganism, the level of reporter gene expression may vary among the different cultures, leading to inconsistent sensitivities (Gupta et al. 2019). Furthermore, microorganisms are capable of rapid evolution in response to environmental changes. Thus, with prolonged usage, the sensitivity and selectivity of the biosensor might be altered (Cui et al. 2019). This might make microbial biosensors somewhat ill-suited for long-term monitoring of bioremediation.

5.7.3 Reliance on Genetic Manipulation When Designing

Most naturally occurring microorganisms are not ideal for the construction of biosensors. Therefore, certain characteristics of these microorganisms are required to be genetically manipulated to achieve the desired properties. Particularly the development of optical microbial biosensors heavily relies on the introduction of reporter genes via genetic engineering techniques (Gupta et al. 2019). This process introduces additional steps which can be time-consuming and expensive. Furthermore, it is challenging to achieve long-term genetic stability of the foreign gene expression.

5.7.4 The High Cost of Development and Maintenance

The development of microbial biosensors requires extensive research on the microorganisms as well as the instrumentations. This requires specialized facilities and human resources, which can be quite costly. Furthermore, the need for genetic

alterations also increases the expense in the construction of microbial biosensors. The use of living microorganisms in microbial biosensors can also add to the maintenance cost of these instruments.

5.8 Future of Microbial Biosensors

The development and application of microbial biosensors have been on the rise during the past few years. One such development was a novel micro-chemostat platform that can be incorporated with microbial biosensors (Bae et al. 2018). Such designs allow the microbial culture to have a uniform environment that ensures long-term stability. In addition, other mechanisms to facilitate the continued stability of the microbial culture environment have also been proposed. These include remote monitoring of cellular and environmental parameters as well as self-stabilizing culture systems (Khire et al. 2020). Another ambitious prospect of microbial biosensing is to develop biosensors that can detect a wide range of signals in a well-coordinated manner. These biosensors may incorporate many microbial species and utilize genetically engineered microorganisms (Gupta et al. 2019).

This chapter mainly focuses on the use of transcriptional regulator/inducible promotor pairs in the design and fabrication of microbial biosensors for monitoring the bioremediation process. In addition, microbial biosensors are designed employing the riboswitch coupled to a reporter gene and the quorum sensing mechanism (Park et al. 2013). For example, an *E. coli* biosensor was designed linking thymidylate synthase with an anti-theophylline aptamer to monitor the theophylline concentration. The concentration of theophylline was monitored based on the dose-dependent repression of GFP expression by theophylline. Similarly, *E. coli* has been engineered to identify specific pathogens via sensing small diffusible molecules involved in the quorum-sensing through reporter gene expression (Park et al. 2013). Although both examples mentioned above are applied in medical diagnosis, it is clear that the underline principle of such biosensors can possibly be used to develop novel microbial biosensors to monitor the bioremediation process. Microfluidics and nanofabrication are another important aspect that has greatly contributed to the development of high throughput microbial biosensors. For example, microbial biosensors integrated with a centrifugal microfluidic platform reduce the time and resources required for analysis while retaining the analytical ability and enhancing portability. Incorporating nanofabrication into microfluidics allows further miniaturization of the microbial biosensor. For instance, microfluidic devices with separate and parallel channels could be designed using soft lithography techniques to screen different toxic compounds enabling high throughput assay on a chip (Lim et al. 2015). Further advances in microbial biosensors can be made by using a panel of bioreporter strains with the same output reporter protein but with different specificities (Van Der Meer and Belkin 2010). Built on this idea, a live cell array was designed in a silicon chip using *E. coli umuDp–lucFF* SOS reporter strain (Tani et al. 2007). Similarly, an array of *E. coli* heat shock responsive reporter cells

was immobilized in micro-electrode chambers (100 nL each) to assess the water toxicity (Popovtzer et al. 2005). Here, silicon-based nano-bio chip, *E. coli* MC1061 that is genetically engineered to elicit a detectable electrochemical signal in the presence of the targeted toxicant was integrated into the nano volume electrochemical cells. The electric current generated by the cells was subsequently analyzed to trace the toxicant level in wastewater in less than 10 min (Popovtzer et al. 2005). In addition to microfluidics, another research group designed a paper-based biosensor to detect and monitor pathogenic bacteria using a quorum sensing mechanism (Brooks and Alper 2021). This paper-based biosensor can be presented as a convenient platform due to low production costs, portability and simplistic manufacturing process.

Microbial biosensors stand out as a promising tool for monitoring the efficacy of the bioremediation process. Further development in genomic and transcriptomic data, protein engineering, computational designing and simulation can accelerate the tailoring of microbial biosensors with better specificity and enhanced performance. Research and development in standardized and streamlined engineering methods will enable the design of more versatile genetic designs and immobilization techniques for real-time high throughput monitoring of the bioremediation process. We can anticipate increased adoption of better tailored, highly sensitive advanced microbial biosensors to effectively monitor the bioremediation process in near future.

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